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Cross-Packaging of Human Immunodeficiency Virus Type 1 Vector RNA by Spleen Necrosis Virus Proteins: Construction of a New Generation of Spleen Necrosis Virus-Derived Retroviral Vectors†

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The ability of the nonlentiviral retrovirus spleen necrosis virus (SNV) to cross-package the genomic RNA of the distantly related human immunodeficiency virus type 1 (HIV-1) and vice versa was analyzed. Such a model may allow us to further study HIV-1 replication and pathogenesis, as well as to develop safe gene therapy vectors. Our results suggest that SNV can cross-package HIV-1 genomic RNA but with lower efficiency than HIV-1 proteins. However, HIV-1-specific proteins were unable to cross-package SNV RNA. We also constructed SNV-based *gag-pol* chimeric variants by replacing the SNV integrase with the HIV-1 integrase, based on multiple sequence alignments and domain analyses. These analyses revealed that there are conserved domains in all retroviral integrase open reading frames (*orf*), despite the divergence in the primary sequences. The transcomplementation assays suggested that SNV proteins recognized one of the chimeric variants. This demonstrated that HIV-1 integrase is functional in the SNV *gag-pol orf* with a lower transduction efficiency, utilizing homologous (SNV) RNA, as well as the heterologous vector RNA of HIV-1. These findings suggest that homology in the conserved sequences of the integrase protein may not be fully competent in the replacement of protein(s) from one retrovirus to another, and there are likely several other factors involved in each of the steps related to replication, integration, and infection. However, further studies to dissect the *gag-pol* region will be critical for understanding the mechanisms involved in the cleavage of reverse transcriptase, RNase H, and integrase. These studies should provide further insight into the design and development of novel molecular approaches to block HIV-1 replication and to construct a new generation of SNV-based vectors.

Retroviral vectors derived from spleen necrosis virus (SNV) and the closely related avian reticuloendotheliosis virus strain A (REV A) have genus-specific relationships with mammalian type C viruses (48). They are nonpathogenic in humans and are capable of delivering exogenous genes into various human cells pseudotyped with targeted envelopes (20, 38, 39). Thus, these retroviral vectors provide an opportunity for their use in human gene therapy. We have tested the ability of the nonlentiviral retrovirus SNV proteins to cross-package the viral (genomic) RNA of the distantly related lentivirus human immunodeficiency virus type 1 (HIV-1) and vise versa. Of note, it has been known that the cross-packaging depends on nucleocapsid (NC) sequences, recognition of the primer-binding sequence (PBS), the polypurine tract (PPT), reverse transcriptase (RT), the attachment sites (*att*), and the integrase (IN) itself for the integration of proviral DNA into the host genome (2, 8, 24, 25, 37).

Integration is a complex multistep process catalyzed by the retroviral IN protein (17, 25, 40). It is an obligatory step for retroviral DNA integration in their respective host cells for efficient replication. Generally, the integration process requires discrete biochemical steps, and the first step involves assembly of the stable complex at long terminal repeats (LTRs) of proviral DNA. The next step is the removal of dinucleotides at 3′ ends, leaving recessed CA dinucleotides at the end of viral DNA, and finally the transport of viral DNA from cytoplasm to nucleus to join the host cellular DNA to become a part of the host genome (1, 12). Each of these stages requires the appropriate assembly of IN protein on viral DNA. Most retroviral INs have three highly conserved individual domains (1). The N-terminal or zinc-binding domain contains His2 Cys2, which suggests interactions with nucleic acid. The central domain is the catalytic core domain, and studies with chimeric HIV-1 IN have shown that this domain is particularly responsible for the selection of nonviral target DNA sites (23, 26–29, 46). There are three amino acids in this domain that are highly conserved among retrotransposon and retroviral INs (15, 16, 21, 32, 49). The third domain is entitled the DNA-binding domain or minimal binding domain and is located at the C′-terminal end of most of the retroviral INs.

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† This study is dedicated to the memory of Ralph Dornburg.

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HIV-1 IN is composed of 288 amino acids (35). The structural domains of HIV-1 IN have been identified by X-ray crystallography and nuclear magnetic resonance spectroscopy. However, catalytic activity with the full-length viral DNA substrate has not yet been elucidated. Thus, the complexity of the IN molecule, coupled with partial knowledge of the functions and specificity of its individual domains, have hampered structure-based rational drug design (3, 21, 22, 36).

Based on our new cross-encapsidation data, we hypothesized that the reduced transduction efficiency during the cross-packaging of HIV-1 RNA by SNV proteins may be related to the efficiency of SNV IN protein. To test the hypothesis that SNV IN is involved in the efficiency of cross-packaging, the SNV IN sequence was compared to other known retroviral IN proteins, including HIV-1, through a computer-based multiple alignment program CLUSTAL (11), and from the databases available on public domains (http://www.ebi.ac.uk/swissprot/). A brief strategy of these analyses is detailed in these studies. Based on multiple sequence analyses and alignment knowledge, a series of chimeric recombinants of SNV *gag-pol*, substituted with or without HIV-1 IN, were designed and constructed. The interactions between HIV-1 RNA and SNV proteins were examined in cross-complementation assays by utilizing the HIV-1 and SNV genomic RNAs. The compatibility of replaced IN protein was also tested by the ability of these viral constructs to integrate and replicate in the target cells.

Furthermore, we were able to utilize these new constructs to define the IN, and indirectly the RNase H domains of SNV (computer-based domain analysis) by using three different regions of SNV *gag-pol* packaging constructs pZP^{32} and $pRDM^{7}$ to fuse HIV-1 IN (37). It has been documented that RNase H is responsible for the hydrolysis of the RNA-DNA hybrid (44) and inactivation of RNase H leads to a noninfectious virus, making this enzymatic activity a target for therapy of HIV-1 infection (19). The study of such chimeric recombinant variants may allow us to probe their diverse functions and lead to the design of novel molecular approaches to block HIV-1 replication. These studies should also further enable the understanding of the molecular pathogenesis of HIV-1 and allow molecular modeling and design of in vitro assay systems in the search for integration inhibitors.

MATERIALS AND METHODS

Plasmid constructions. Retroviral vector pZP^{32} is an SNV-based REV A (Gag) packaging construct. The SNV *gag* (334 bp) was replaced by the REV A *gag* (342 bp) with nuclear localization signal sequences within the SNV *pol*, as described previously (38, 39) (Fig. 1A). This plasmid utilizes the cytomegalovirus (CMV) promoter, followed by the adenovirus tripartite leader sequences (AVt l) for enhanced gene expression. We have constructed a series of SNV-based HIV-1 IN chimeric packaging constructs by replacing the SNV IN sequence with HIV-1 IN, using computer-based alignment and modeling information. The construct $pSHI\text{-}ZP^{1}$ was generated by removing 733 bp (AgeI and SalI sites) from the 3′ end of *pol* region of the SNV packaging construct, pZP^{32} (Fig. 1B and 4) and was substituted by HIV-1 IN in the translational *orf* of the SNV *gag-pol* ($pRDM^{7}$) (38). A second construct, $pSHI\text{-}ZP^{2}$, was created by deleting five amino acids from (4989 to 4600 nucleotides [nt]) the distal end of RNase H adjacent to the herpes simplex virus type 1 protein-like UL14 region, as noted in the postulated sequence map of SNV *pol* (Fig. 1B). The five-amino-acid deletion was generated by amplifying a fragment of 381 bp between 4604 and 4985 nt (fragment A) from the SNV *gag-pol* region by PCRs (Fig. 1) by using oligonucleotides IN A (sense; 5′-ACTCTACACGTCCATGGGAT-3′; nt 4600 to 4620) and IN C (antisense; 5′-TCTAAAATCCCCACTGCTCT-3′; nt 4990 to 4976]) with HIV-1 IN flanking sequences. The HIV-1 IN was amplified as fragment B of 862 bp (4228 to 5093 nt of pNL4-3) by using the IN D sense oligonucleotide (5′-AGTGGGGATTTTAGATGGATT-3′) and the IN B antisense oligonucleotide (5′-GTCGACAATCCTCATCCTGTCTACTG-3′) (underscored letters represent HIV-1 nucleotides). The 5′-flanking sequences of HIV-1 IN, as well as the SNV *pol* regions, were annealed (A+B) and then amplified by PCR. The resulting 1,261-bp fragment was cloned into pGEM-T Easy vector (Promega, Madison, Wis.) for sequence verification before cloning the DNA fragment back into pZP^{32} by using its AflIII and SalI sites. The chimeric variant $pSHI\text{-}ZP^{3}$ was constructed by deleting three amino acids from the middle of RNase H region of $pSHI\text{-}ZP^{2}$ by using the NsiI and SalI sites. To abolish the SNV IN activity, a chimeric variant, pS-I, was constructed by deleting a 311-bp fragment from the middle of SNV IN without altering the other *gag-pol* polyproteins. Restriction sites AgeI(5371) and HindIII(5682) were used, and the cleaved ends were filled in before blunt-end ligation. All of these constructs were created through several steps of cloning. The purpose of these constructs was to test the ability of SNV and HIV-1 IN chimeric proteins to integrate homologous and heterologous RNA (Fig. 1B) and to clearly define the respective boundaries of the SNV IN, as shown by the postulated sequence map.

SNV-based transfer vector pZP^{36} (Fig. 1A) was derived from pAK3 (derived from pO11) by the addition of the CMV promoter at the XhoI site in the U3 region of the 3′ LTR through a XhoI linker (39). The β-galactosidase (β-Gal) gene was cloned as a marker gene by using XbaI and HindIII sites (Fig. 1A). pJD220SVHy is an SNV-based transfer vector containing an internal simian virus 40 (SV40) promoter expressing the hygromycin phosphotransferase B gene (*hygro*) (13, 14).

HIV-1 packaging and transfer constructs. The packaging construct pCMVΔR8.2 was kindly provided by Didier Trono (University of Geneva) and has been described previously (34). The transfer vectors pHR′ CMV LacZ and pHR′CMV Puro are described elsewhere (34, 51). These two constructs were used in the *trans*-complementation assays along with packaging construct. The vesicular stomatitis virus envelope protein G (VSV-G) expression construct, pMD.G, was used in all of the experiments and has been described previously (34).

Transfections and transductions. The 293T cells were maintained at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The canine osteosarcoma cell-line (D17), permissive for the SNV replication or infection, was maintained at 37°C in DMEM supplemented with 7% calf serum (BioWhittaker, Baltimore, Md.). PC-3 is a cell-line derived from human prostate cancer cells procured from the American Type Culture Collection (Manassas, Va.) and was maintained at 37°C in DMEM supplemented with 10% fetal bovine serum. All cell lines were maintained under standard tissue culture conditions. For transfection assays, 293T cells at 70 to 80% confluency were cultured in six-well plates, and each transfection was carried out by using FuGene 6 reagent (Roche Molecular Diagnostics, Alameda, Calif.) according to the manufacturer's protocol. The DNA concentration of each construct was normalized before transfection studies. The target cell lines—D17, 293T, and PC-3—were plated 18 h prior to the infection. The viral supernatants were collected 72 h posttransfection, and cellular debris was removed by centrifugation at 2,000 × *g* for 10 min. The viral supernatants were then divided into three equal parts, and one part was used for the infection. The target cells were exposed to supernatant in the presence of 25 μg of Polybrene/ml for 4 h. The cells were then washed with 1× phosphate-buffered saline (PBS) to remove the virus and supplemented with fresh medium. After 48 h, the cells were washed and stained for β-Gal activity as a marker gene with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as described previously (38). The blue cells

TABLE 1. Cross-packaging of SNV and HIV-1 RNAs[a]

Triple transfection assay (plasmid)	Virus titer (CFU/ml) in cell line:		
	293T	D17	PC-3
SNV *gag-pol* + SNV transfer vector	0.5×10^4	1.0×10^4	0.5×10^3
SNV *gag-pol* + HIV-1 transfer vector	1.0×10^2	0.5×10^2	1.0×10^2
HIV-1 *gag-pol* + SNV transfer vector	<10	<10	<10
HIV-1 *gag-pol* + HIV-1 transfer vector	2.0×10^4	0.5×10^4	1.0×10^5
SNV transfer vector (control)	Nil	Nil	Nil
HIV-1 *gag-pol* (control)	Nil	Nil	Nil

[a] 293T cells were transfected with three plasmids from each virus (see Materials and Methods for details). At 48 h posttransfection, supernatants were collected, and viral particles harvested for the transduction of various cell lines. The HIV-1 packaging construct and SNV transfer vectors were transfected as controls. Nil, less than two in a 60-mm plate.

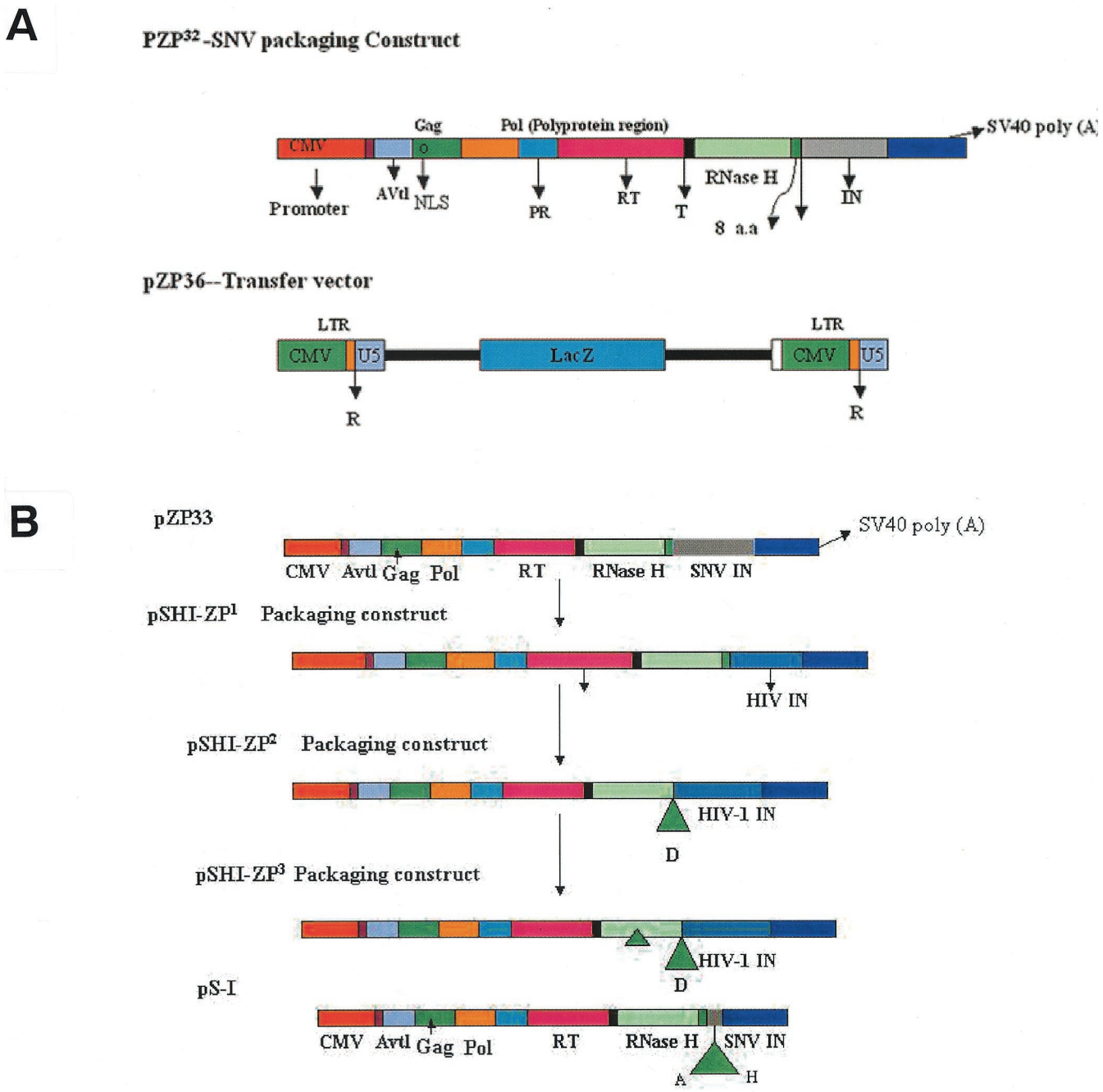

FIG. 1. (A) Schematic representation of the SNV packaging and transfer vectors used in the cross-packaging of HIV-1 RNA into SNV particles and vice versa. The SNV-based packaging construct pZP[32] with REV A Gag and SNV Pol polyproteins are illustrated. Regions of the plasmids are as follows: CMV, CMV promoter/enhancer; Avtl, avian tripartite leader sequence; NLS, nuclear localization sequence; Gag (REV A), Gag polyproteins of reticuloendotheliosis virus; PR, protease; T, tether; RNase H; IN, integrase; SV40 [poly(A)], the polyadenylation sequence derived from SV40 early promoter; and the SNV-based transfer vector PZP[36]. The U3 region was replaced with human CMV promoter to be expressed in human cells (R and U5). Parts of SNV LTRs included LacZ and the bacterial LacZ gene. (B) Three SNV-based chimeric variants derived from pZP[32] packaging construct, substituted with HIV-1 IN. pSHI-ZP[1] was generated by replacing the SNV IN with HIV-1 IN by multiple sequence alignment. pSHI-ZP[2] was derived from pSHI-ZP[1] by deleting eight amino acids from the junction between the RNase H and IN sequences. pSHI-ZP[3] was constructed by the additional deletion of three amino acids from the pSHI-ZP[2] within the RNase H region by using the postulated sequence map of SNV *gag-pol*. D, deletion. pS-I was generated by deleting a 311-bp fragment from the middle of SNV IN by using AgeI and HindIII sites (details are provided in Materials and Methods).

from each transduction experiment with LacZ-containing virus were scored to determine the titer in CFU/milliliter (Table 1) (39). The cells infected with antibiotic-resistant virus were kept under selection for another 10 days. The remaining viral stocks were either used to detect the HIV-1 p24 antigen by enzyme-linked immunosorbent assay (ELISA) or pelleted by ultracentrifugation at 65,000 × *g* (Beckman ultracentrifuge model L7/rotor NVT90) at 4°C for 2 h. The pelleted virus was then resuspended in 100 μl of TNE buffer (50 mM Tris-Cl [pH 7.4], 100 mM NaCl, 1 mM EDTA [pH 8.0]) and stored at −80°C for RNA isolation. The antibiotic-resistant colonies were expanded, harvested, and pooled to isolate the genomic DNA, by using a genomic DNA isolation kit (Promega)

according to the protocol described by the manufacturer. The extracted genomic DNA was used to evaluate the integrated viral DNA in host genome by PCR techniques. The primers specific for hygromycin and puromycin markers genes were used to evaluate integrated viral DNA (data not illustrated).

Viral and cellular RNA isolation and slot blot analysis. The transfected cells were washed with 1× PBS after the removal of supernatant and lysed directly on the plates by using the RNAqueous- 4-PCR kit (Ambion, Inc., Austin, Tex.) according to the manufacturer's protocol. The harvested viral particles were resuspended in 100 μl of TNE buffer and used to extract the RNA with the RNAqueous 4-PCR kit. The extracted RNA was treated with RNase-free DNase to remove any DNA contamination. The integrity of the RNA was examined by gel electrophoresis and spectrophotometrically. The slot blot apparatus was used to analyze the cellular and viral RNA for the presence of a LacZ marker gene, as described previously (33, 34). Equal amounts of the viral and cellular RNA were transferred to the Zeta membrane (Bio-Rad Laboratories, Hercules, Calif.) and fixed on a slot blot apparatus as recommended by the manufacturer. The membrane was washed in transfer buffer and allowed to dry and then UV cross-linked for 30 s. The transferred RNA sample membrane was prehybridized with North2South buffer (Pierce Biotechnology, Rockford, Ill.) at 55°C for 1 h. The membrane was then hybridized for 4 h with LacZ probe (310-bp PCR-amplified fragment) labeled with [α-^{32}P]dATP by using a Random-Prime-a-Gene kit (Promega). The forward and reverse primers used for amplification of the *lacZ* gene were 5′ TGGATAACGACATTGGCGTAA 3′ and 5′ TG GACGCGGTCGACCGTCAAG 3′ respectively. The membrane was washed with 0.1% SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C, air dried, and exposed for 1 h using Kodak X-ray film. Quantification of the RNA and hybridization was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and ImageQuant (IQMac 1.2V software).

Amplification of integrated proviral DNA. The possibility of cross-contamination of vector DNA was ruled out by using SNV transfer vector pJD220Hy in place of pZP^{36}, and pHR′CMV Puro in place of pHR′CMV LacZ. The antibiotic-resistant colonies were expanded, and the cellular lysate of individual colonies was used for PCR with hygromycin- and puromycin-specific primers (data not illustrated).

RESULTS

SNV Proteins can cross-package and propagate genomic HIV-1 RNA. To analyze the ability of SNV proteins to cross-package heterologous genomic HIV-1 RNA, we utilized the SNV packaging construct pZP^{32} (expressing *gag-pol*) to cross-package HIV-1 RNA generated from the pHR′CMV *LacZ* vector (34), as well as homologous (SNV) RNA generated from the pZP^{36} transfer vector (control) in a triple plasmid transfection assay. The G protein of VSV (pMDG) (33, 34) was utilized as the envelope of choice. The retroviral particles generated from these transfection experiments were used to infect various cell lines, such as human prostatic carcinoma (PC-3), dog osteosarcoma (D17), and human embryonal kidney (293T), for titer determinations. The overall results of in vitro transductions involving SNV proteins to package homologous SNV RNA were titers of 0.5×10^4 for 293T cells and of 1.0×10^3 and 0.5×10^3 for the D17 and PC-3 cell lines, respectively. There was a 100-fold decrease (1.0×10^2) in titer when SNV proteins were used to cross-package HIV-1 RNA to infect 293T and D17 cells. The titer was only 10-fold lower (1.0×10^4 to 1.0×10^2) in PC-3 cells (Table 1). However, when HIV-1 packaging proteins were used to cross-package SNV RNA, the transduction results clearly indicated a nonreciprocal packaging in all of the cell lines. Nonetheless, the viral titer was 100-fold increased (2.0×10^4) when HIV-1 RNA genome was packaged by its own proteins, as opposed to the SNV proteins (1.0×10^2). The PC-3 cell line showed the exception with a 10-fold further decrease in titer (1.0×10^5 to 1.0×10^2), as illustrated in Table 1. Comparison of these analyses revealed that HIV-1 RNA was equally stable and efficiently expressed in the presence of SNV protein complementation. Alternatively, the cross-packaging efficiency of HIV-1 RNA by SNV proteins was 100-fold lower (determined through staining of the marker gene) and cell type dependent, as opposed to homologous SNV or HIV-1 RNA packaged by their own proteins. The infection assay revealed no transduction when the SNV *gag-pol* construct pS-I was cotransfected with the SNV transfer vector, ensuring no integration (Table 1).

HIV-1 core protein P24 antigen ELISA. The cell lysates and supernatants harvested from the 293T cells, transfected either with the HIV-1 *gag-pol* construct alone (pHR′CMΔR8.2), HIV-1 *gag-pol* with the HIV-1 transfer vector, or with the HIV-1 *gag-pol* construct and SNV transfer vector (pZP^{36}), were analyzed for HIV-1 p24 antigen by ELISA. The results revealed a higher level of p24 antigen production in cell lysate from the cross-complementation of HIV-1 *gag-pol* with SNV RNA (data not illustrated), in contrast to the cells expressing only HIV-l proteins, or HIV-1 proteins with HIV-1 RNA. The cell-free p24 antigen level was also analyzed from these supernatants. Our results revealed a higher p24 antigen level from the supernatant generated with HIV-1 proteins alone, as opposed to when HIV-1 proteins were either packaged with HIV-1 RNA or cross-packaged with SNV RNA. These results suggest that the cell-associated p24 antigen level was not blocked by SNV genomic RNA. The p24 antigen production over time was also evaluated from the supernatants of infected target cells. These studies showed no sign of further production of p24 antigen during or after the infection in any of the experiments (data not illustrated). Together, these results demonstrated enhancement of p24 antigen levels by the SNV genome despite no cross-packaging, and the data were further supported by the infectivity assays (Table 1).

Nonreciprocal cross-packaging of SNV RNA by HIV-1 protein. The ability of HIV-1 proteins to cross-package SNV RNA was determined by using the HIV-1 packaging construct pHR′CMVΔR8.2 (33) and the SNV transfer vector pZP^{36} (SNV LTRs) carrying β-Gal as a marker gene, plus pMDG (VSV-G) as the envelope of choice in triple transfection assays. The virus-containing supernatant was harvested, and the cellular debris was removed by centrifugation and used to infect target cells, isolate RNA, and obtain p24 antigen ELISA determinations. After the removal of the supernatant, the transfected cells were washed with 1× PBS, and the total cellular RNA was isolated. The first-strand cDNA was generated from 1 μg of RNA from each sample. The LacZ-specific primers were used to amplify each cDNA for the presence of SNV vector RNA and were evaluated by gel electrophoresis to demonstrate efficient intracellular expression of vector RNA, as illustrated in Fig. 2A (see lane 4). The cellular and virion RNA were then hybridized with the LacZ-specific [α-^{32}P] dATP-labeled probe in a slot blot assay, as illustrated in Fig. 2B, slots B and B′, and in Fig. 2C. The total cellular RNA hybridization results revealed that the intracellular SNV transfer vector RNA was efficiently expressed, although the packaging was altered. The quantity of RNA was not decreased, even after 48 h (data not illustrated), compared to the level of RNA generated from the homologous species. However, when the RNA isolated from the same supernatant was hybridized with the LacZ probe, the results showed no packaging of SNV RNA by HIV-1 proteins, despite efficient expression of vector

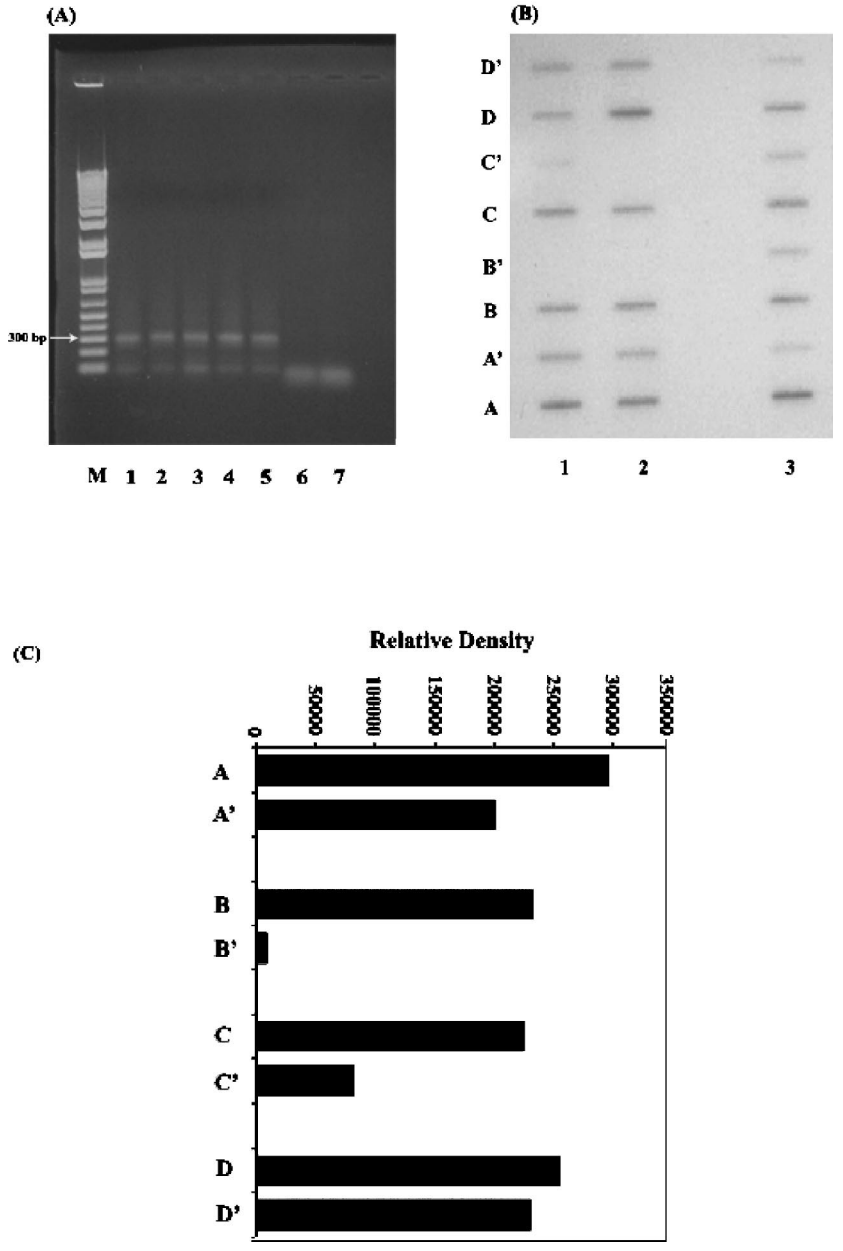

FIG. 2. (A) RT-PCR of cell-associated viral vector RNA isolated from transfections used in cross-packaging studies. cDNAs were amplified by a pair of LacZ primers with a 310-bp amplicon by using equal concentrations of RNA. Lanes: M, 1 kb plus marker DNA; 1, PCR amplification of cDNA generated from the transfected cells with pZP[36]; 2, cDNA amplification of cellular RNA from SNV packaging construct plus SNV transfer vector; 3, SNV packaging construct plus HIV-1 transfer vector; 4, HIV-1 packaging construct plus SNV transfer vector; 5, HIV-1 transfer construct plus HIV-1 transfer vector RNA; 6, PCR amplification of cellular RNA with the SNV transfer construct alone (control); 7, SNV packaging construct plus HIV-1 transfer vector, with the same primers to rule out the possibility of DNA contamination (control). (B) Slot blot analysis of cellular and virion RNAs. The cellular and viral RNA from each transfection was hybridized with α-^{32}P-labeled LacZ DNA probe synthesized by PCR. Slots 1A and 2A show 0.5 μg of cellular RNA blotted from 293T cells transfected with SNV packaging and transfer vectors. Slot 3A shows 0.1 μg of cellular RNA from the DSH.cxl cell line, as a control RNA. Slots 1A′ and 2A′ show 0.5 μg of virion RNA from the SNV control experiments (SNV packaging with SNV transfer vector), followed by the 3A′ slot carrying 0.1 μg of virion RNA from the DSH.cxl cell line (titer of 10^6/ml). Slots 1B and 2B represent the cellular RNA from cross-packaging of SNV RNA by HIV-1 proteins. Slot 3B shows cellular RNA from the DSH.cxl cell line. Slots 1B′ and 2B′ show the virion RNA from cross-packaging SNV RNA and HIV-1 proteins, with slot 3B′ showing the control virion RNA. Slots 1C and 2C show 0.5 μg of cellular RNA from SNV proteins cross-packaging HIV-1 RNA; slots 1C′ and 2C′ represent 0.5 μg of virion RNA from the SNV proteins cross-packaging HIV-1 RNA. Slots 3C and 3C′ show cellular and virion RNAs from the DSH.cxl cell line, respectively. For D and D′, slots 1 and 2 represent cellular and virion RNA from the HIV-1 control experiment (HIV-1 proteins with HIV-1 RNA), respectively, whereas slots 3D and 3D′ are cellular and virion RNA from the DSH.cxl cell line. (C) Quantitative data representing cellular and viral RNA by PhosphorImager analyses. A and A′ show cellular and virion RNA from SNV packaging and transfer vectors, respectively. B and B′ show cellular and virion RNA generated by SNV proteins complemented with the HIV-1 transfer vector, respectively. C and C′ show cellular and virion RNA from HIV-1 proteins with SNV RNA, respectively. D and D′ show cellular and virion RNA from HIV-1 proteins with HIV-1 RNA, respectively.

TABLE 2. SNV chimeric variants with homologous and heterologous RNAs[a]

Complementation assay (gag-pol$^+$ VSV-G) construct	Virus titer (CFU/ml) in 293T cells with:	
	SNV RNA (pZP^{36}) (homologous RNA)	HIV-1 RNA pHR′CMV LacZ (heterologous RNA)
SNV pZP32 (control)	1.0×10^4	1.0×10^2
pSHI-ZP^1	1.0×10^2	1.0×10^2
pSHI-ZP^2	<10	<10
pSHI-ZP^3	Nil	Nil
pS-I	Nil	Nil
pHR CMVΔR8.2	Nil	1.2×10^4

[a] The infectivity of four chimeric variants, along with vectors for two control experiments, was determined by using 293T cells in a triple-transfection assay. The virus was harvested, and target cells were transduced. The titer was determined by measuring β-Gal expression in the target cells. Nil, less than two in a 60-mm plate.

RNA. This suggests that there was no reciprocal cross-packaging by HIV-1 proteins with SNV RNA (see Fig. 2B, slot B′, and C). The virion, as well as the cellular RNA isolated from the cross-packaging of HIV-1 RNA by SNV proteins, demonstrated RNA expression and capability of packaging by the SNV proteins in the form of particles (Fig. 2B, slots C and C′, and Fig. 2C). Similar results were revealed by transduction assays with less efficiency (Table 1), suggesting the cross-packaging capability of SNV proteins for HIV-1 RNA.

Individual hygromycin- and puromycin-resistant colonies containing proviral DNA. To rule out the possibility of plasmid DNA cross-contamination and to ensure the successful transfer of HIV-1 RNA or SNV RNA in retroviral particles, the cells were selected with hygromycin or puromycin-containing medium after infection. The colonies were grown and expanded for PCR analyses to confirm the presence of hygromycin- or puromycin-resistant genes (data not illustrated). We detected no hygromycin-selected colonies with the HIV-1 RNA, as well as in SNV JD220SVHy hygromycin RNA experiments. However, puromycin-resistant colonies were demonstrated with the SNV proteins and the HIV-1 RNA transcomplementation assay, in addition to the hygromycin- and puromycin-resistant colonies obtained from the control experiments. These results revealed that there was no packaging of SNV RNA by HIV-1 particles. SNV proteins are capable of cross-packaging HIV-1 RNA, showing nonreciprocal cross packaging.

Replacement of SNV IN by HIV-1 IN based on computer-predicted sequence analysis. The postulated boundaries of the SNV IN domain as shown in Fig. 1 were removed and replaced by the HIV-1 IN sequence, except in construct pS-I (inactive IN), and each construct was used in cross-complementation assays to copackage HIV-1 RNA or SNV RNA. The function of the IN was tested by integration of the LacZ marker gene in the target cells. The infected cells were stained with a β-Gal solution, and the titer was determined by counting the blue cells as CFU (CFU/ml). The results of these SNV chimeric proteins with HIV-1 fused IN are presented in Table 2. These results revealed that HIV-1 IN is functional in a SNV IN location within the *gag-pol orf* (sequence map, Fig. 1B), with a titer of 10^2 CFU/ml in the cross-complementation assay when we used packaging construct pSHI-ZP^1 and HIV-1 heterologous and SNV homologous RNA. These results also suggest that there was not significant difference in the titers, even after substitution with HIV-1 IN protein. However, there was a 10-fold further decrease in the titer (in CFU/milliliter) with the recombinant construct pSHI-ZP^2 cross-packaging (Table 2), as well as in utilizing the homologous RNA. However, the construct pSHI-ZP^3 was unable to integrate into the host DNA, demonstrating no cross-packaging of heterologous RNA. The construct pS-I was unable to transduce any cell type, suggesting no integration due to the lack of IN activity (see Table 2). Together, these results suggest that SNV proteins are capable of cross-packaging the HIV-1 RNA, and HIV-1 IN is functional in the SNV *gag-pol* region, although the low packaging efficiency of homologous and heterologous RNA remained unchanged.

DISCUSSION

SNV proteins are capable of cross-packaging HIV-1 genomic RNA. The present study demonstrates the surprising finding that SNV can cross-package the RNA of HIV-1, a more distantly related retrovirus. We used several complementary experimental approaches to ensure that SNV structural proteins were capable of packaging HIV-1 vector RNA in single rounds of replication. The viral titer or the transduction results from the cross-packaging of heterologous RNA of HIV-1 showed a 100-fold reduction compared to the viral titers of control experiments of homologous SNV or HIV-1 virus (Table 1). The data was further substantiated by slot blot hybridization of viral and cellular RNA. The hybridization results were than quantified by using a PhosphorImager, as illustrated in Fig. 2C. These results revealed a 2.5-fold reduction in the viral mRNA generated by the copackaging of HIV-1 transfer vector by the SNV proteins, as opposed to the viral mRNA produced by SNV transfer vector utilizing its own proteins. However, there was a 3.5-fold viral mRNA reduction, compared to when HIV-1 proteins were complemented with HIV-1 transfer vector. The viral mRNA generated by HIV-1 proteins cross-packaging the SNV RNA genome was undetectable as quantified by PhosphorImager (Fig. 2C). These results were further supported by infection assays, illustrated in Table 1. One possible explanation for this low titer could be the less-efficient packaging of HIV-1 RNA by SNV proteins. It has been documented that SNV Gag proteins interact with a hairpin motif signal sequence and, therefore, may have a nonpreferential packaging for HIV-1 RNA (9). The results from viral RNA slot blot hybridization assays further supported the cross-packaging of HIV-1 RNA by SNV proteins, as illustrated in Fig. 2B, slots C and C′.

It was also observed that HIV-1 proteins were unable to reciprocate SNV RNA packaging, as shown by *trans*-complementation assays (Table 1). RT-PCR results revealed that the expression of SNV RNA was equally efficient and stable, as shown in Fig. 2A, lane 4. This nonreciprocal SNV RNA packaging may be a result of poor utilization of the RNA into viral particles compared to the others (Fig. 2A, lanes 2 and 5). Similar results were demonstrated by the analysis of genomic DNA from the infected cells when they were subjected to PCR with the LacZ primers. The slot blot hybridization of these isolated RNA indicated that cell-associated viral vector RNA was generated from each transfection assay and was hybridized with the LacZ probe. These data are supported by other stud-

ies (4, 6, 9, 30), suggesting that the level of reciprocal cross-packaging is mostly dependent on the stringent requirements of packaging signals and their recognition by the packaging proteins (6, 11, 16).

Nonreciprocal RNA packaging by HIV-1 proteins. The cell-associated HIV-1 p24 antigen ELISA showed production of p24 core proteins by the cross-packaging of SNV vector RNA with HIV-1 proteins (data not illustrated). The p24 antigen levels were increased in cross-packaging experiments compared to cells transfected with the HIV-1 *gag-pol* and HIV-1 vector RNA. There was a decrease in p24 antigen when HIV-1 proteins were solely expressed. The cell-free p24 antigen released by HIV-1 proteins alone was slightly higher compared to in the HIV-1 control experiment (HIV-1 vector RNA and HIV-1 proteins). The cell-free p24 antigen level was increased when HIV-1 proteins were solely expressed, as opposed to when complemented with SNV RNA (data not shown).

The p24 antigen data suggest that HIV-1 proteins were expressed but were unable to interact with SNV RNA to be utilized for packaging into particles, thus showing a higher value of p24 core proteins as opposed to the HIV-1 proteins expressed and utilized when HIV-1 RNA was packaged into particles. Our results are in agreement with the findings of Boris-Lawrie and coworkers (7, 42). Those studies relate the high production of p24 antigen to the 5′ RNA terminus of SNV, which stimulates the translation of nonviral mRNA of HIV-1 by facilitating the nuclear export of rev responsive element-dependent mRNA, thus enhancing the expression of Gag protein (7). Despite the high expression of HIV-1 Gag proteins, there was no noticeable transduction observed (Table 1 and Fig. 2A, lane 4, and B, slot B′). The p24 ELISA time courses were also performed after the target cells were infected and, surprisingly, no further production of p24 core proteins was observed after one round of replication. These results were further supported by the data obtained from infection studies. This clearly indicates that the SNV RNA was generated and HIV-1 packaging proteins were expressed, as determined by p24 antigen ELISA, and yet there was no packaging of RNA. As noted above, these results were further supported by viral RNA slot blot hybridization assays, illustrated in Fig. 2B, slots B and B′, thus supporting a nonreciprocal preference by HIV-1 proteins for SNV RNA. Our data are also in agreement with those of Browning et al. (6), which revealed that feline immunodeficiency virus and HIV-1 RNAs were able to cross-package and propagate each other's RNA, whereas a distantly related viral RNA was unable to reciprocate this effect (9), suggesting that the RNA from the same viral group can be efficiently packaged with structural proteins of the other related viruses (18).

Sequence analyses of the SNV *pol* region utilized to propose an SNV IN model. Our studies provide insights into the cross-packaging of HIV-1 by SNV proteins and nonreciprocal packaging of SNV RNA by HIV-1 proteins, suggesting that the mechanisms used to achieve packaging in each virus differ. The relatively low packaging efficiency of SNV proteins for HIV-1 RNA may be explained by the inability of SNV polyproteins to support HIV-1 viral replication, which may require additional specific factors besides the packaging proteins (9). Several previous studies have suggested that NC sequences and *cis*-acting factors, such as primer-binding sites, polypurine tracts, the *att* sequences, RT, and IN, are possible factors involved in virus-specific packaging, integration, and replication (6, 9).

To address these questions, we compared the *cis*-acting sequences of the SNV and HIV-1. We found only 2 nt homologous (CA) to the HIV-1 3′*att* sites, compared to SNV. These sequences are recognized by IN for the integration of preintegration complexes into the host DNA. Previous studies revealed that not only the homology but the size of the sequence also matters, suggesting that differences in the sizes of SNV *att* sites may also be involved in cross-packaging (6). We explored the sequence homology of the SNV *pol* region, specifically the IN, with other known and well-defined IN proteins, including HIV-1 (Fig. 3), by using multiple sequence alignment program CLUSTAL and domain analysis through the SwissProt database. The structure and function of most of the retroviral IN are highly conserved (31, 50). We then compared the conserved regions of HIV-1 IN with SNV IN to determine the possibility of substituting this *orf* for SNV IN, as illustrated in Fig. 3.

Based on the homology of the SNV IN protein, three different chimeric *gag-pol* expression constructs were created by substituting HIV-1 IN at different positions, while one construct was without IN activity (Fig. 1B). These constructs were used in triple transfection assays against the homologous and heterologous RNAs. The data from our studies clearly indicate that the construct pSHI-ZP[1], with substitution of the HIV-1 IN, showed a packaging efficiency 100-fold less than the HIV-1 or SNV proteins packaging their own RNA. However, there was no difference in the cross-packaging efficiency of HIV-1 RNA compared to the homologous SNV RNA, as shown in Table 2 (30). One possible explanation could be the difference in the size of the *att* between these two viruses. The difference in NC sequence between HIV-1 and SNV proteins could be another factor involved in cross-packaging. This may be the reason why pSHI-ZP[1] showed the same transduction efficiency by utilizing homologous as well as heterologous RNA, since there was HIV-1 IN present but there were different *att* and NC sequences (Table 2) (8, 30). These results also suggest that substitution of HIV-1 IN could not improve the cross-packaging efficiency, in contrast to the studies performed by Liu et al. (31), wherein the infection was increased by replacing HIV-1 IN by HIV-2 IN in cross-complementation. The construct pSHI-ZP[2], with HIV-1 IN, along with a deletion of four amino acids adjacent to RNase H region, was in the correct translational *orf* frame of Pol polyproteins, yielding surprising results by failing to integrate into the host DNA and showed 10-fold further reduction in infection of the target cells. The other construct pSHI-ZP[3] was derived from pSHI-ZP[2] by an additional deletion of three amino acids from the RNase H region and failed to transduce target cells. The construct pS-I expressing an inactive IN was also unable to transduce the target cells, indicating the failure of integration. These results indicate that the HIV-1 IN either failed to cleave from the rest of the polyproteins or may have a different route for intracellular folding. A further reason may be the deletion of five amino acids from the *pol* region which are necessary for the hydrolysis of the RNA-DNA hybrid. The deletions may have hampered its activity and the RNA-DNA hybrid may be unable to separate for integration into the host genome. However, this possibility has not been fully elucidated (5, 10, 47).

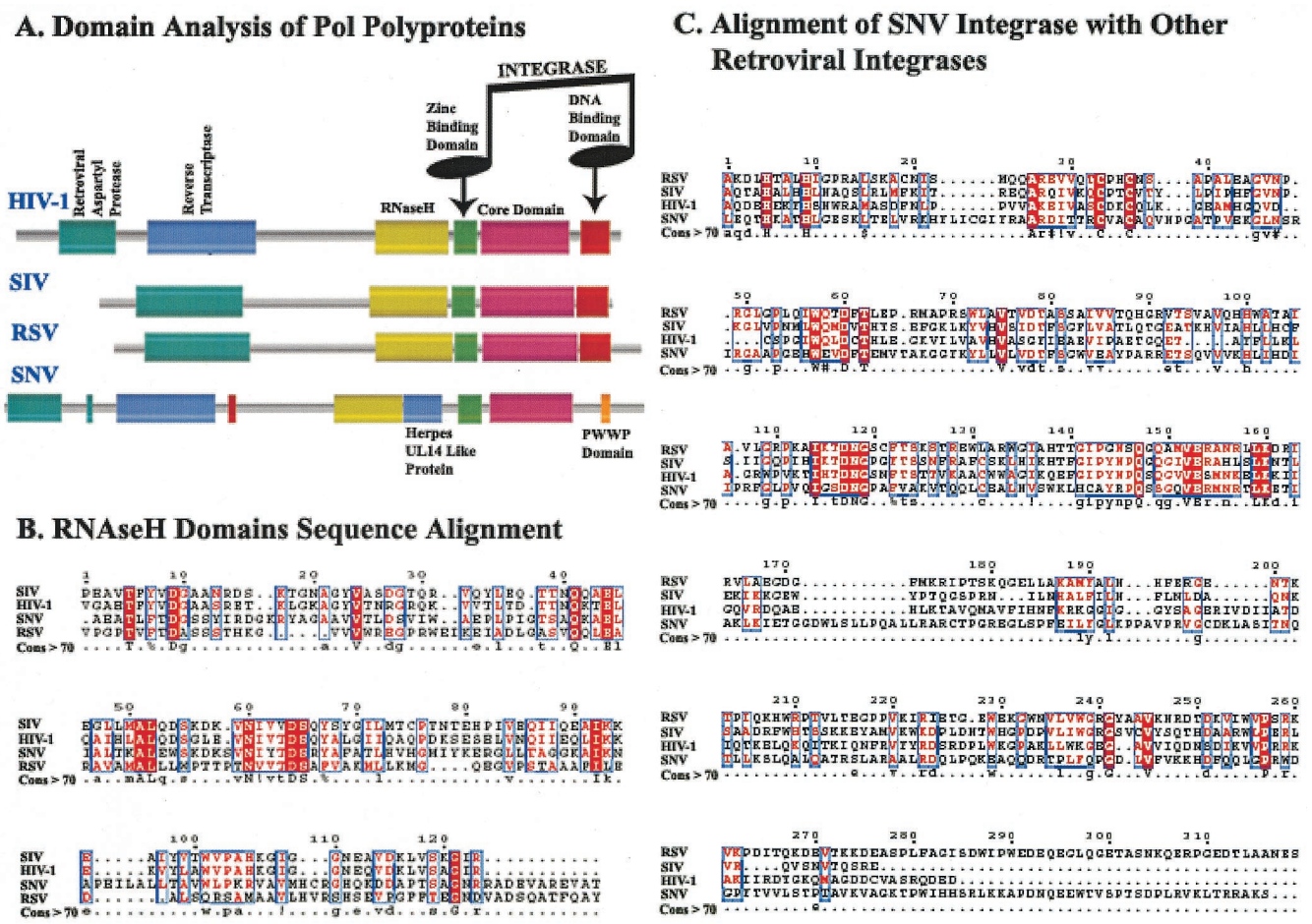

FIG. 3. Domain analysis and alignment of the Pol protein of SNV and other closely related retroviruses for defining the boundaries of SNV regions. (A) Boundaries of various domains of SNV *Pol*; (B) RNase H sequence alignment of SNV with SIV, HIV-1, and RSV; (C) alignments of SNV IN with closely related retroviral INs.

Russell et al. (43) investigated the ability of human foamy virus IN to integrate circular DNA and could not detect any significant increase in the level of integration. Similarly, the infection studies from these two constructs indicated that the IN either failed to integrate DNA or became inactive after further deletions in the *pol orf* (Table 2). This inability could be related to several factors, such as the involvement of RNase H in hydrolyzing the RNA-DNA hybrid or the possibility that a frameshift mutation in the *orf* occurred (5, 10, 19, 41, 44, 45). This could result in a premature termination of Pol polyproteins. Another factor could be that the protease cleavage site used by SNV IN was lost, and HIV-1 IN was unable to exhibit its activity after the removal of part of the *pol* region necessary for IN cleavage from the polyproteins (37). Further deletion may also have affected the integration capability. The construct pS-I was unable to integrate and showed no transduction (37). These interesting results have led us to hypothesize that HIV-1 IN is functional in the SNV *pol orf* and that the RNase H region is at the 3′ end of the *pol*, before the IN.

In summary, our study clearly demonstrates that the packaging specificities of two retroviruses, SNV and HIV-1, are quite different. SNV proteins are more flexible and less signal sequence specific than HIV-1 proteins, which are very selective (9). Thus, this nonreciprocal cross-packaging between the two viruses somewhat diminishes safety concerns regarding the use of the SNV as retroviral vectors for human gene therapy protocols. There would be less of a chance of recombination between these viral species. The study also suggests that replacement of homologous sequences from one virus to another virus is likely to evolve a dysfunctional characteristic in the chimeric virus, as we found in two of the chimeric variants of SNV. The present data provide novel information by examining the chimeric viruses, and further studies to dissect the chimeric variants with HIV-1 IN may help elucidate molecular approaches for the development of inhibitors of the replication and integration processes.

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